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Neutrophil-activating peptide-2 and processes for the production of NAP-2, B-TG, CTAP-III and PBP

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U1S S2410 S2411

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(continued on next page)

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(58) Field of search

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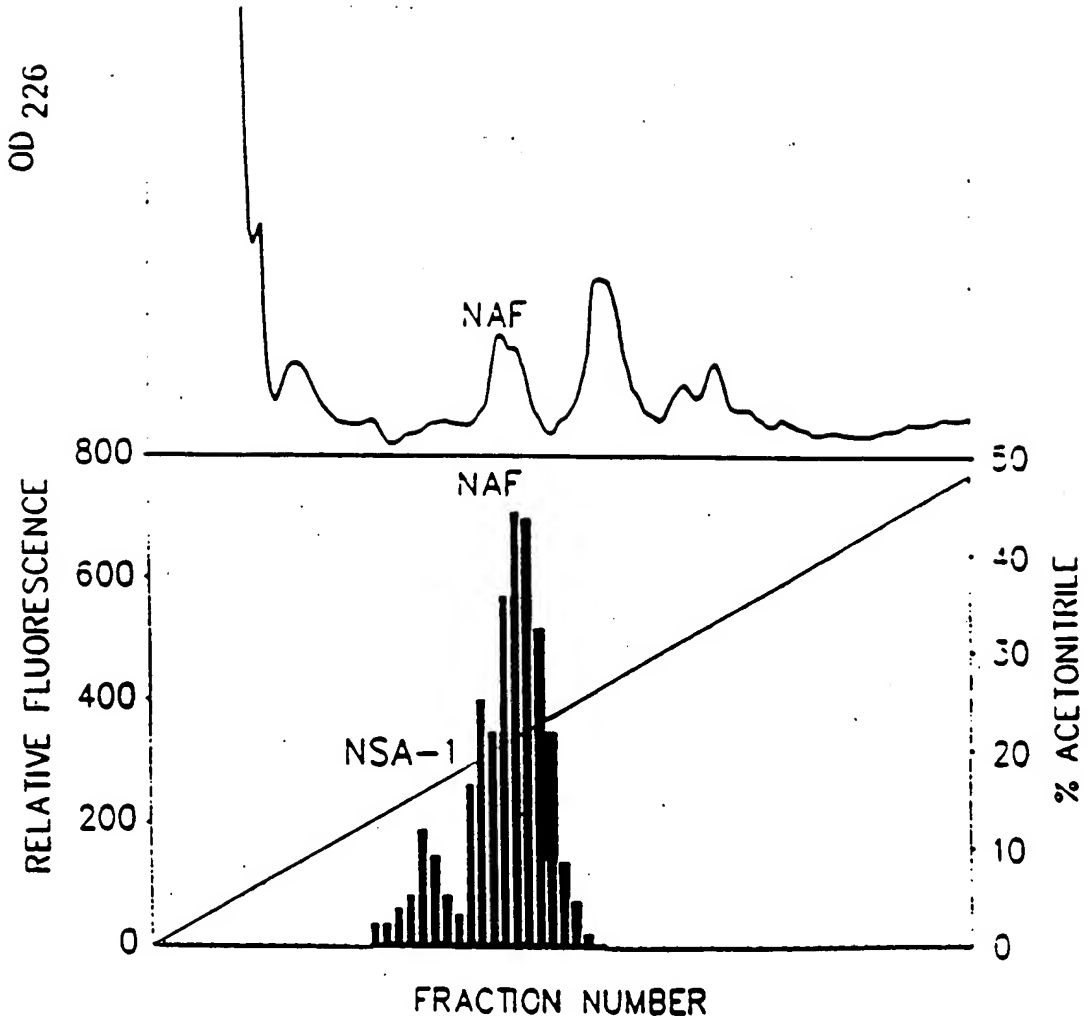
FIG. 1a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NAF
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400
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30
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% ACETONITRILE
FRACTION NUMBER

FIG. 1b

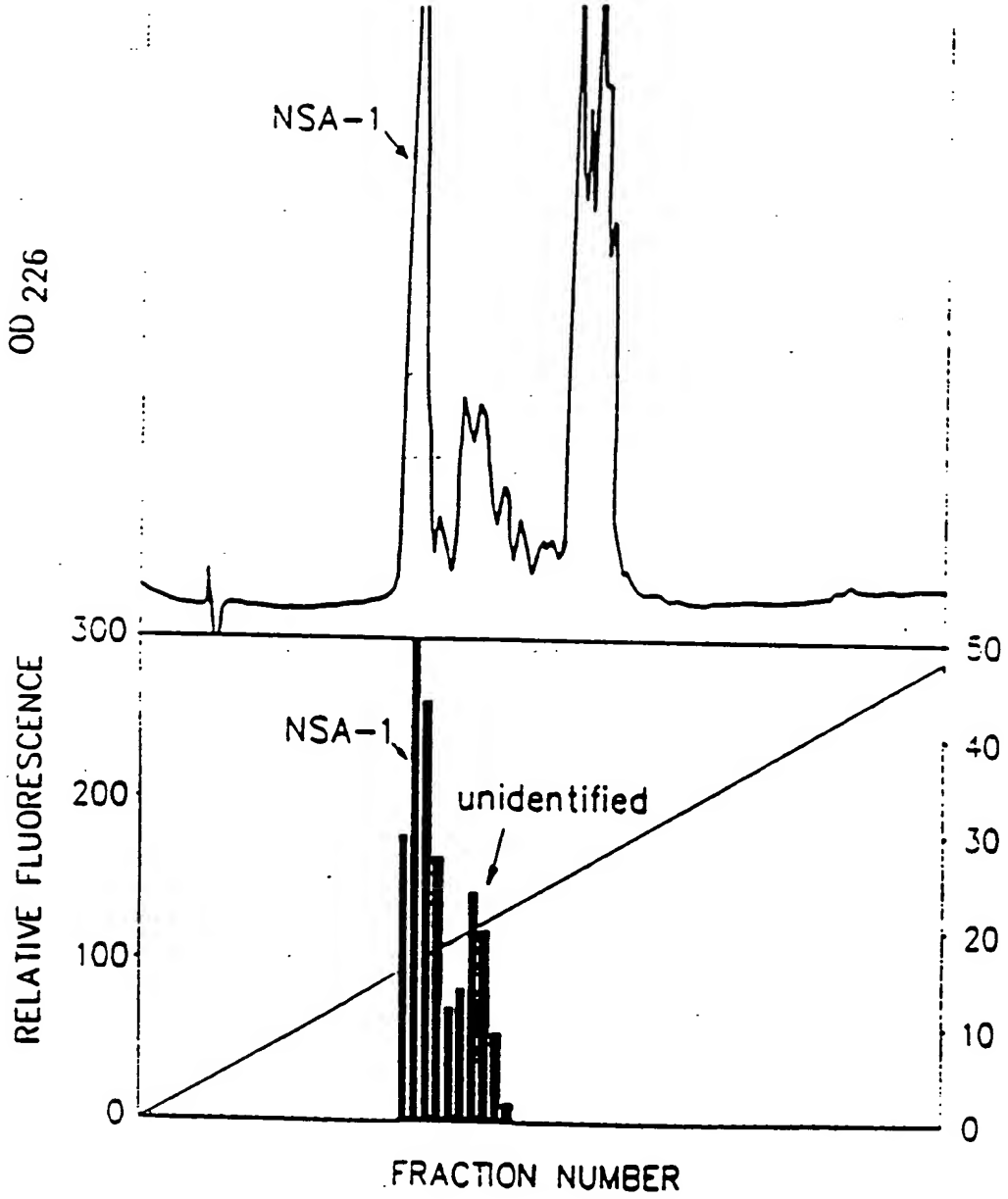
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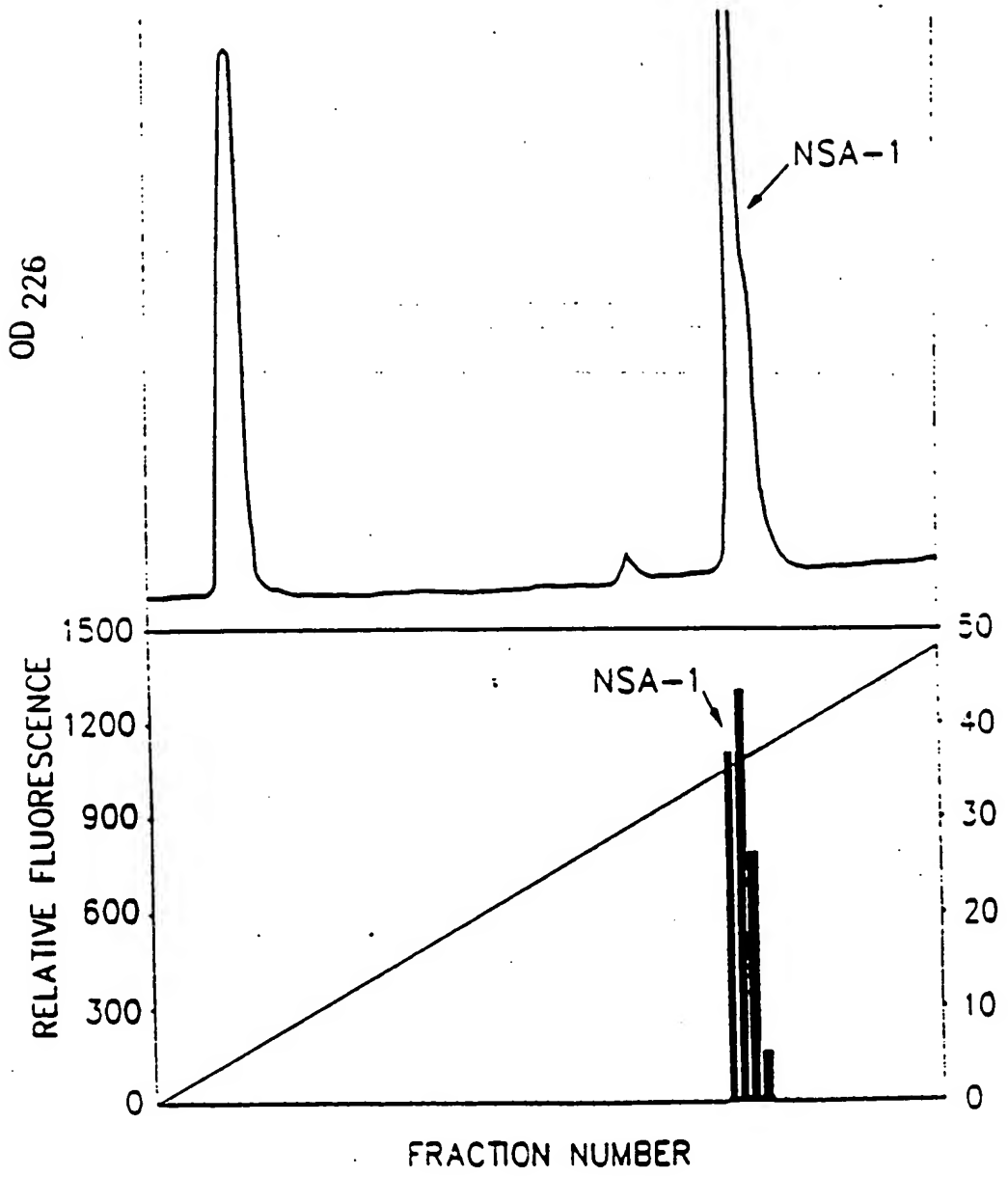
FIG. 1c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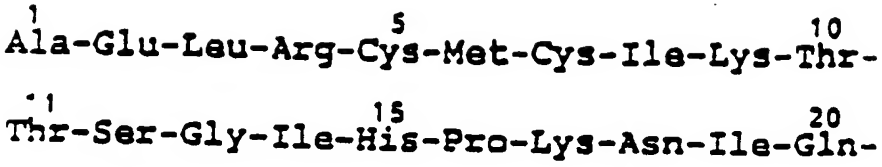
FRACTION NUMBER

Figure 2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Ala-Glu-Leu-Arg-Cys-Met-Cys-Ile-Lys-Thr-
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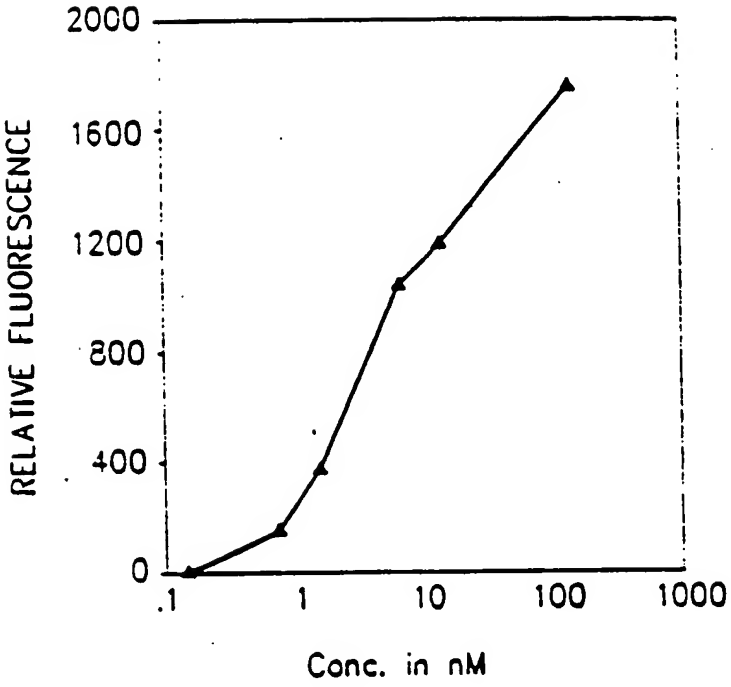
10
Thr-Ser-Gly-Ile-His-Pro-Lys-Asn-Ile-Gln-
15
20

FIG. 3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800
400
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RELATIVE FLUORESCENCE
.1
1
10
100
1000
Conc. in nM

Figure 4

1 5 10
Ala-Glu-Leu-Arg-Cys-Met-Cys-Ile-Lys-Thr-

11 15 20
Thr-Ser-Gly-Ile-His-Pro-Lys-Asn-Ile-Gln-

21 25 30
Ser-Leu-Glu-Val-Ile-Gly-Lys-Gly-Thr-His-

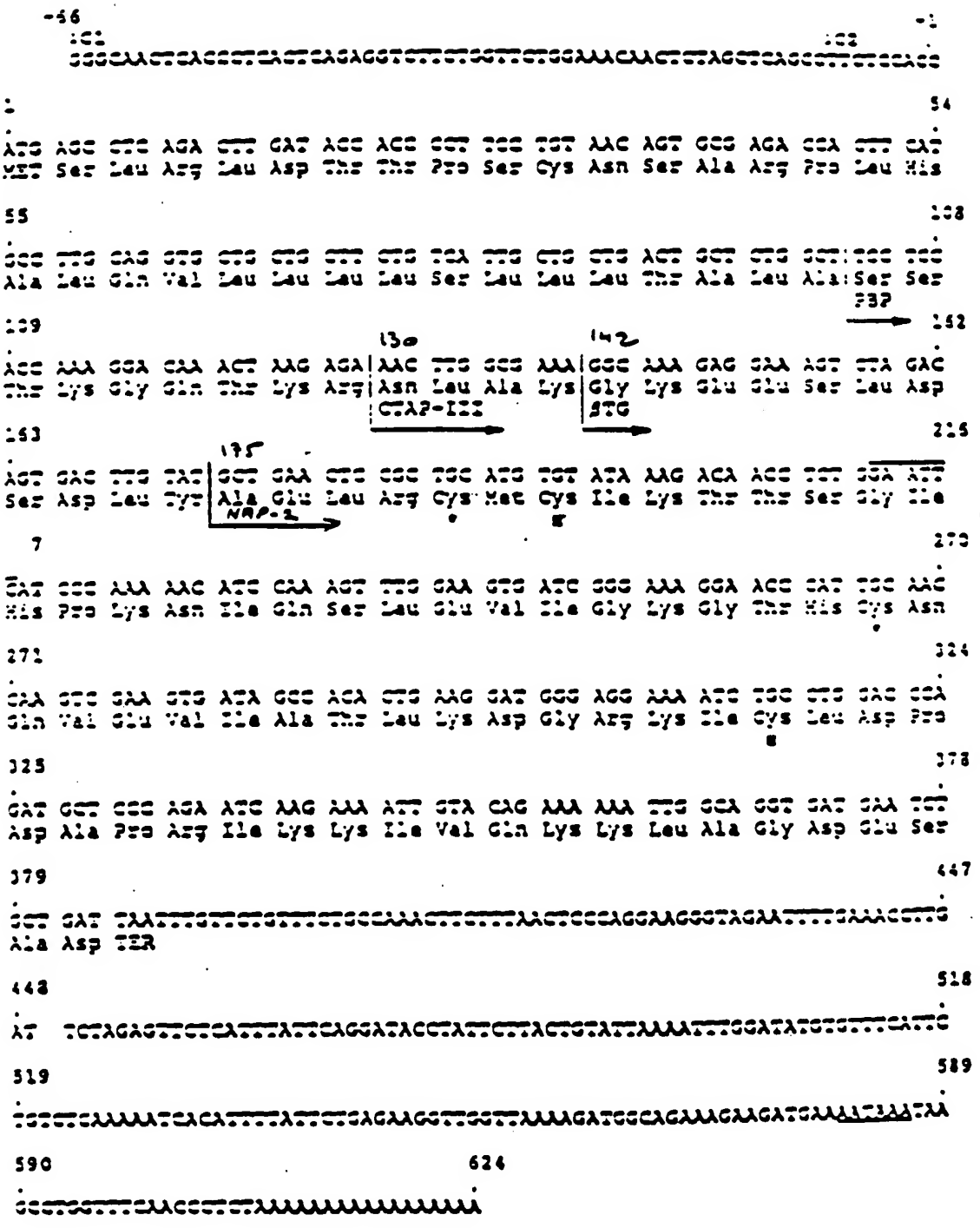
31 35 40
Cys-Asn-Gln-Val-Glu-Val-Ile-Ala-Thr-Leu-

41 45 50
Lys-Asp-Gly-Arg-Lys-Ile-Cys-Leu-Asp-Pro-

51 55 60
Asp-Ala-Pro-Arg-Ile-Lys-Lys-Ile-Val-Gln-

61 65 70
Lys-Lys-Leu-Ala-Gly-Asp-Glu-Ser-Ala-Asp

Figure 5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ICI
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FBP
109
152
130
142
Thr Lys Gly Gln Thr Lys Arg Asn Leu Ala Lys Gly Lys Glu Glu Ser Leu Asp
CTAP-III
GTG
153
215
175
Ser Asp Leu Tyr Ala Glu Leu Arg Cys Met Cys Ile Lys Thr Thr Ser Gly Ile
NAP-2
7
270
His Pro Lys Asn Ile Gln Ser Leu Glu Val Ile Gly Lys Gly Thr His Cys Asn
271
324
Gln Val Glu Val Ile Ala Thr Leu Lys Asp Gly Arg Lys Ile Cys Leu Asp Pro
325
378
Asp Ala Pro Arg Ile Lys Lys Ile Val Gln Lys Lys Leu Ala Gly Asp Glu Ser
379
447
Ala Asp TER
448
518
519
589
590
624

NEUTROPHIL-ACTIVATING PEPTIDE-2 AND PROCESSES FOR THE PRODUCTION OF NAP-2, B-TG, CTAP-III AND PBP

The present invention is concerned with an immuno-modulatory substance.

It is more particularly concerned with an immuno-stimulating factor which activates neutrophil leukocytes, especially human neutrophil leukocytes. It is hereinafter referred to as neutrophil-stimulating activity 1 (NSA-1) or, synonymously, neutrophil-activating peptide 2 (NAP-2).

1. BACKGROUND

The neutrophil leukocytes (neutrophils) are the most common leukocytes and account for about 2/3 of the white cells in human blood. They have one main function which is to protect the host organism against microbial infections. The neutrophils are mobile, responsive to chemotactic stimuli generated upon infection, and are

able to move into infected tissues to kill the microorganisms. The killing depends on the ability of the neutrophils to engulf the microorganisms and to release oxygen radicals and microbicidal enzymes. The release of such products depends on activation of the neutrophils.

A few proteins having such activity are known, such as neutrophil-activating factor (NAF) (P. Peveri et al., J. Exp. Med. 167 [1988] 1547), also termed neutrophil-activating peptide 1 (NAP-1).

2. SUMMARY OF THE INVENTION

It has now been found that neutrophil-stimulating activity is produced by stimulated leukocytes in culture and can be obtained from the culture fluid. The neutrophil-stimulating activity is referred to herein as NSA-1 or, synonymously, neutrophil-activating peptide-2 (NAP-2).

It has also been found that NSA-1/NAP-2 is structurally very similar to β-thromboglobulin (β-TG), connective tissue activating peptide III (CTAP-III) and platelet basic protein (PBP).

It is an object of the invention to provide NSA-1/NAP-2, in a degree of purity sufficient to allow its further characterization and preparation by e.g recombinant DNA techniques, and its pharmaceutical use.

The invention further provides a process for the preparation of NSA-1/NAP-2 from human blood leukocytes and/or platelets.

It further provides for the use of NSA-1/NAP-2 in activating neutrophil leukocytes and thus enhancing resistance to infections.

It further provides a process for preparing NSA-1/NAP-2, or biologically active β-TG, CTAP-III or PBP by recombinant DNA techniques which comprises cloning a corresponding gene including the natural leader sequence endogenous to human platelets, expressing it in a suitable host and appropriately recovering the peptide product, if indicated, for preparing NSA-1/NAP-2, using an appropriate protease to cleave the resultant β-TG, CTAP-III or PBP.

3. ABBREVIATIONS

BSA	bovine serum albumin;
CTAP-III:	connective tissue-activating peptide III.
DDS:	sodium dodecyl sulfate;
DTT:	dithiothreitol;
fMLP:	N-formyl-L-methionyl-L-leucyl-L-phenylalanine;
LPS:	lipopolysaccharide from E.coli 055:D5;
MEM:	Eagle's minimal essential medium (Seromed GmbH, Munich, FRG), supplemented with 25 µg/ml neomycin, buffered to pH 7.4 with 25 mM $NaHCO_3$ and 20 mM HEPES;
MEM-PPL:	contains in addition 1 % pasteurized plasma protein solution (5 % PPL-SRK, Swiss Red Cross Laboratory, Bern, Switzerland) and 100 IU/ml penicillin and streptomycin (Gibco AG, Basel, Switzerland);
MoAbs:	monoclonal antibodies;
mRNA:	messenger RNA;
NAF:	neutrophil-activating factor (= NAP-1);
NAP-1:	neutrophil-activating peptide 1 (= NAF);
NAP-2:	neutrophil-activating peptide 2 (= NSA-1);
NEM:	N-ethyl maleimide;
NSA-1:	neutrophil-stimulating activity 1 (= NAP-2);

PBP: platelet basic protein;

PBS: phosphate-buffered saline without Ca^{++} and Mg^{++};

PBS-BSA: PBS supplemented with 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$ and 2.5 mg/ml BSA;

PRP: platelet-rich plasma;

PHA-P: phytohaemagglutinin (Difco Laboratories, Detroit, MI, USA)

PMN: polymorphonuclear cells - neutrophils

PMSF: phenylmethane sulfonyl fluoride;

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis;

SSPE: 130 mM NaCl; 10 mM NaH_2PO_4; 1 mM EDTA; pH 7.4;

β-TG: β-thromboglobulin.

4. DETAILED DESCRIPTION

NSA-1/NAP-2 is characterized biologically by its neutrophil-activating properties, in particular the induction of granule enzyme release. In molecular terms NSA-1/NAP-2 is characterized by a molecular weight of approximately 7500 and a calculated isoelectric point of approximately 8.7.

It is produced from e.g. human blood leukocytes and/or platelets by a process comprising purification from culture fluids of stimulated blood leukocytes and/or platelets by phosphocellulose chromatography and reversed-phase chromatography.

NSA-1/NAP-2 from other species than human may be produced in similar manner from corresponding blood leukocytes and/or platelets.

Stimulation may be effected with any known agent such as LPS and PHA-P.

Phosphocellulose chromatography may e.g. be effected on a phosphocellulose column equilibrated with a potassium phosphate/NaCl/EDTA/glycerol buffer at approximately neutral pH, e.g. pH 7.2, and subsequent elution in e.g. a linear NaCl concentration gradient in the same buffer.

Reversed-phase chromatography normally follows phosphocellulose chromatography and preferably is effected first on a preparative reversed-phase C4 column eluted with e.g. a 0 to 80 % gradient of acetonitrile in 0.1 % trifluoroacetic acid; then on a CN-propyl column eluted with e.g. a gradient of 0-80 % acetonitrile in 0.1 % trifluoroacetic acid; and thereafter the active fractions are rerun on an analytical reversed-phase C4 column under conditions similar to those used for the CN-propyl chromatography.

The course of the purification may be seen from Figures 1a, 1b and 1c.

The course of the purification is followed by analysis for neutrophil-stimulating activity, e.g. as the capacity to induce release of elastase from human neutrophils pretreated with cytochalasin B (B. Devald and M. Baggiolini, Biochem. Pharmacol. 36 [1987] 2505-2510).

The NSA-1/NAP-2 is found to have an apparent molecular weight of approximately 6500 upon 20 % urea - SDS polyacrylamide gel electrophoresis. The apparent isoelectric point is about 8.3.

Amino acid sequence analysis shows (Figure 2) that the first 20 N-terminal amino acids correspond exactly to a common portion of the sequence of platelet basic protein (PBP) and its structural derivatives CTAP-III (C.W. Castor et al., PNAS 80 [1983] 765-769) and β-thromboglobulin (G.S. Begg et al., Biochemistry 17 [1978] 1739-1744). The amino-terminus of NSA-1/NAP-2 corresponds to amino acid 16 of CTAP-III and amino acid 12 of β-TG. Digestion with carboxypeptidase Y shows that the C-terminal amino acid sequences are identical (-Glu-Ser-Ala-Asp). NSA-1/NAP-2 aligns completely to the sequence of β-TG and consists of 70 amino acids with a calculated molecular weight of 7628 and a calculated isoelectric point of 8.7. The full 70 amino acid sequence is shown in Figure 4. The overall homology between NSA-1/NAP-2 and NAF/NAP-1 is 46 %. The NSA-1/NAP-2 sequence does not contain any apparent sites for N-glycosylation. There is a potential site for phosphorylation by protein kinase C (Thr) at position 39 and a possible amidation site (Asp) at position 42.

NSA-1/NAP-2 can thus be vieved as being a fragment of β-TG.

The sequence of NSA-1/NAP-2 can be aligned to that of NAF/NAP-1 on the basis of the two first cysteine residues (Cys 5 and Cys 7 for NSA-1/NAP-2 and Cys 7 and Cys 9 for NAF/NAP-1). When aligned in this way, about one half of the first 20 amino acids of NSA-1(NAP-2 and NAF/NAP-1 are identical. The two factors are thus related not only functionally but also to a certain degree structurally.

The availability of the amino acid sequence of NSA-1/NAP-2 allows its preparation by further processes in addition to the isolation from a natural source described above. Thus, since the peptide includes only 70 amino acids, total synthesis is possible in conventional manner, e.g. using the Merrifield solid state method and having due regard to the presence of two disulfide bonds.

Further production processes include recombinant DNA techniques, e.g. by cloning and expression of a corresponding synthetic gene, optionally after codon optimization. The chemical synthesis and expression in yeast of a gene encoding CTAP-III has been described (G.T. Mullenbach et al., J. Biol. Chem. 261 [1986] 719). However, only part of the CTAP-III produced had biological activity. It seems likely that misfolding and incorrect disulfide-bond formation were major problems. No DNA coding for a leader sequence was incorporated in the synthetic gene since no such leader sequence was yet known for this class of compounds.

A further method of production by recombinant DNA techniques is the cloning and expression of a gene including the natural leader sequence endogenous to human platelets, e.g. by cloning and expression of a cDNA selected from an appropriate expression library and coding for NSA-1/NAP-2 or a larger peptide encompassing NSA-1/NAP-2 such as β-TG, CTAP-III or PBP and recovery of NSA-1/NAP-2 in conventional manner from the expression product. The leader sequence is the first 34 amino acids in the sequence of Figure 5.

Example 7 describes the cloning of a cDNA coding for CTAP-III from a human platelet-derived λgt11 expression library. Recovery of the desired peptide product from a larger parent peptide is effected by appropriately truncating the larger peptide in conventional mannner with a protease such as a serine protease.

Appropriate proteases may e.g. be isolated in conventional manner from purified monocytes. They are highly sensitive to PMSF, moderately sensitive to leupeptin and insensitive to EDTA.

Alternatively, the leader sequence may be directly attached to the gene coding for the desired peptide.

Preparation via cloning of a gene including a natural leader sequence results in peptide products having the proper folding for full biological activity, e.g. for targeting to the α-granule, when expressed in mammalian cells.

Three variants of NSA-1/NAP-2 with somewhat reduced biological activity have also been found, having the 70 amino acid sequence shown in Figure 4 but elongated at the N-terminus by the 3, 4 and, respectively, 5 corresponding amino acids of CTAP-III (see Figure 5). They thus have the sequence of Figure 4 preceded by, respectively:

Asp-Leu-Tyr-,

Ser-Asp-Leu-Tyr- and

Asp-Ser-Asp-Leu-Tyr-

and are of 73, 74 and, respectively, 75 amino acids in length.

They can be produced as described above for the preparation of NAP-2 from stimulated blood leukocytes and/or platelets: upon stimulation with LPS in the presence of monocyte culture supernatant, in addition to NAP-2 a smaller intermediate peak containing the 73-75 residue variants of NAP-2 is obtained. This additional peak is made up to 65, 20 and 15 percent by the 74, 75 and 73 residue form, respectively.

NSA-1/NAP-2 and functional variants,

thereof possess biological activity making them indicated for use as pharmaceuticals.

For example, they elicit neutrophil infiltration in rats in dosage of from about 1 ug/kg to about 100 ug/kg animal body weight.

NSA-1/NAP-2 and functional variants,

thereof are thus indicated for use in the treatment of conditions which are accompanied or caused, locally or systemically, by a modification of the number or activation state of the PMN (polymorphonuclear cells - neutrophils). They extensively modify these PMN parameters and are therefore indicated for use in the treatment of conditions in which an increase of the number or enhancement of the activation state of the PMN leads to clinical improvement, e.g. in bacterial, mycoplasma, yeast and fungal, and in viral infections. Furthermore, they are indicated for use in inflammatory illnesses such as psoriasis, arthritic conditions and asthma, or in conditions of abnormally low neutrophil count and/or generalized low neutrophil level, and in the preparation of antagonists, e.g. monoclonal antibodies, for use in these indications.

Since, as NAP-1, they have been shown to be also chemotactic for T-lymphocytes, they are also indicated for use in certain immunodeficient states.

For these indications the appropriate dosage will, of course, vary depending upon, for example, the host, the mode of administration and the nature and severity of the condition to be treated. However, in general, satisfactory results are indicated to be obtained systemically at daily dosages of from about 1 mg/kg to about 100 mg/kg animal body weight. For the larger subject an indicated daily dosage is in the range of from about 0.1 mg to about 100 mg, preferably from about 0.1 mg to about 10 mg, conveniently administered, for example, in divided doses up to four times a day.

Pharmaceutical compositions comprising the compound in association with at least one pharmaceutically acceptable carrier or diluent can be manufactured in conventional manner by mixing with a pharmaceutically acceptable carrier or diluent. Unit dosage forms contain, for example, from about 0.025 mg to about 50 mg of the compound.

While functionally largely similar to NAP-1, NSA-1/NAP-2 can probably only be generated in vivo when PBP and/or CTAP-III are liberated from platelets, while the production of NAF/NAP-1 by mononuclear phagocytes and a wide variety of tissue cells is induced by inflammatory cytokines like tumor necrosis factor and interleukin-1. The two peptides must, therefore, be expected to arise in dissimilar physiological situations and at different sites. Being platelet derived, NAP-2 is produced mainly intravascularly, where platelet activation and aggregation occurs, e.g. in thrombi and atherosclerotic lesions, while NAF/NAP-1 almost invariably forms in the tissues.

NSA-1/NAP-2 and its variants are not found in platelets or other components of the mononuclear cell cultures, and appear to be formed following release. They exhibit the typical properties of chemotactic receptor agonists and induce cytosolic free calcium changes, chemotaxis and exocytosis in the same molar range as NAF/NAP-1, while PBP, CTAP-III and PF-4 have little if any activity at 100 to 10000 times higher concentrations. They are expected to be as effective as NAF/NAP-1 and C5a in the recruitment of neutrophils, and to have a role in thrombosis, where they could attract neutrophils involved in the recanalization of obstructed vessels.

5. EXPLANATION OF THE FIGURES

Figure 1a: Preparative reversed-phase high pressure liquid chromatography of NSA-1 on a C4 column. Upper graph: protein distribution, OD at 226 nm; lower graph: NSA-1 activity, relative release of elastase from human neutrophils.
Two peaks are evident: a small one corresponding to NSA-1/NAP-2 and a larger one corresponding to NAF/NAP-1.

Figure 1b: Reversed-phase high pressure liquid chromatography of NSA-1/NAP-2 on a CN-propyl column. Details as in Figure 1a.

Figure 1c: Reversed-phase high pressure liquid chromatography of NSA-1/NAP-2 on a C4 column. Details as in Figure 1a.

Figure 2: Amino-terminal sequence of NSA-1/NAP-2. The first 20 residues are shown. They correspond to part of the sequence of β-thromboglobulin.

Figure 3: NSA-1/NAP-2-induced exocytosis in cytochalasine B - treated human neutrophils. Concentration dependance.

Figure 4: Amino acid sequence of NSA-1/NAP-2.

Figure 5: cDNA sequence and deduced amino acid sequence of the precursor of PBP (starts with nucleotide 103), CTAP-III (starts with nucleotide 130), β-TG (starts with nucleotide 142) and NSA-1/NAP-2 (starts with nucleotide 175). The two internal disulfide bonds are identified with asterisks and squares respectively. The putative polyadenylation signal is underlined (nucleotides 581-586). The EcoRI recognition site is overlined.

6. EXAMPLES

The following Examples illustrate the invention.

PART 1: PRODUCTION OF NSA-1/NAP-2 FROM HUMAN BLOOD, PURIFICATION AND CHARACTERIZATION

Example 1: Production of NSA-1/NAP-2 by human blood leukocytes and/or platelets stimulated with LPS

Anticoagulated donor blood, obtained from the Swiss Red Cross Laboratory and stored for up to 20 hours at 4-10°C was used. Mononuclear cells (consisting of monocytes and lymphocytes in a ratio of approximately 1:5) were isolated from single buffy coats on Ficoll-Hypaque (RTM) gradients (A. Boyum, Scand. J. Immunol. 5 [1976] 9-15), and were washed in MEM. The washed cells from 6 buffy coats were resuspended in MEM-PPL ($5x10^6$ cells/ml) and cultured for 20 hours in the presence of 1 µg/ml LPS in glass bottles equipped with a stirring device. At different times, aliquots of the culture media were sampled and the neutrophil-stimulating activity was determined as the capacity to induce release of elastase from human neutrophils pretreated with cytochalasin B (B. Devald and M. Baggiolini, Biochem. Pharm. 36 [1987] 2505-2510). This test detects NSA-1 as well as NAF, a formerly described neutrophil-activating factor (P. Peveri et al., J. Exp. Med. 167 [1988] 1547-1559). Neutrophil-stimulating activity increased with time and levelled off after 24 to 48 hours.

Example 2: Production of NSA-1/NAP-2 by human blood leukocytes and/or platelets stimulated with PHA-P

Anticoagulated donor blood, obtained from the Swiss Red Cross Laboratory and stored for up to 20 hours at 4-10°C was used. Mononuclear cells (consisting of monocytes and lymphocytes in a ratio of approximately 1:5) were isolated from single buffy coats on Ficoll-Hypaque gradients (Boyum, 1976), and were washed in MEM. The washed cells from 6 buffy coats were resuspended in MEM-PPL ($5x10^6$ cells/ml) and cultured for 20 hours in the presence of 5 µl/ml PHA-P in glass bottles equipped with a stirring device. At different times, aliquots of the culture media were sampled and the neutrophil-stimulating activity was determined as the capacity to induce release of elastase from human neutrophils pretreated with cytochalasin B. Neutrophil-stimulating activity increased with time and levelled off after 24 to 48 hours.

Example 3: Purification of NSA-1/NAP-2 from the culture fluids of stimulated human blood leukocytes and/or platelets stimulated with LPS

a) Phosphocellulose chromatography

Portions of 700 ml of cell-free culture fluids of human leukocytes stimulated as described in Example 1 were directly loaded onto a 15 ml phosphocellulose column (Whatman® P11) equilibrated with buffer A (20 mM potassium phosphate buffer, pH 7.2, containing 20 mM NaCl, 1 mM EDTA and 5 % glycerol). The column was washed with the same buffer and then eluted (24 ml/hour) with 120 ml of a linear NaCl concentration gradient (0.02 to 1.5 M) in buffer A. Fractions were analyzed for neutrophil-stimulating activity.

b) Reversed-phase chromatography

Active fractions obtained from the phosphocellulose chromatography separation were pooled and further purified by 4 repeated runs on a wide-pore preparative reversed-phase C4 column (10 x 250 mm, 7 µm, Macherey-Nagel, Dueren, FRG). The column was eluted at 2 ml/min with a gradient of 0 to 80 % acetonitrile in 0.1 % trifluoroacetic acid with an increment of 0.66 % per min.

Active fractions with a retention time of 20-26 min were pooled, concentrated on a Speed Vac (RTM) centrifuge and loaded onto an analytical reversed-phase CN-propyl column (4.6 x 250 mm, 5 µm, wide-pore, Baker Research Products, Phillipsburg, N.J., USA). The column was eluted at 0.5 ml/min with a gradient of 0 to 80 % acetonitrile in 0.1 % trifluoroacetic acid with an increment of 0.66 % per min. Active fractions with a retention time of 22-25 min were pooled, concentrated and rerun on an analytical reversed-phase C4-column (4.6 x 250 mm, 5 µm, wide-pore, Baker Research Products) under the conditions described for the CN-propyl column. Active fractions with a retention time of 42.5 min were dried in a Speed Vac centrifuge, resuspended in sterile water and then used for gas-phase sequence analysis and for biological testing. Figure 1 shows the separation of NSA-1/NAP-2 from NAF/NAP-1 and the fractions used for amino acid sequence analysis.

Example 4: Gel electrophoresis of purified NSA-1/NAP-2

Purified NSA-1/NAP-2 was analyzed on a 20 % urea-SDS polyacrylamide gel according to B. Kadenbach et al., Analyt. Biochem. 129 [1983] 517-521). A single band with an apparent molecular weight of 6500 was obtained upon visualization by silver staining. NAP-2 migrated slightly faster than NAF/NAP-1.

Example 5: Amino acid sequence analysis of NSA-1/NAP-2

Amino acid sequence analysis was performed by automated phenyl isothiocyanate degradation using an Applied Biosystems gas phase sequencer Model 477 A. Samples of NSA-1/NAP-2 (500 pMol) were applied directly or after chemical modification. Reduction and alkylation was performed as follows: 1 nMol of NSA-1/NAP-2 was diluted in 150 µl of 6 M guanidinium hydrochloride, 2 mM EDTA, 0.2 M Tris-HCl, pH 8.3, then 225 nl of tributylphosphine in 15 µl acetonitrile were added and the solution incubated at room temperature. After 60 min 160 nl of 4-vinylpyridine in 10 µl acetonitrile were added. After 30 min an equal amount of tributylphosphine and vinylpyridine was added, and the reaction was continued for another 40 min under nitrogen. The solution was the acidified with trifluoroacetic acid to pH 2.0 and desalted by reversed-phase HPLC in 0.1 % trifluoroacetic acid with an acetonitrile gradient.

The carboxy-terminus was determined with 0.5 nmol of NAP-2 and 0.4 µg of carboxypeptidase P or carboxypeptidase Y (sequencing grade, Boehringer).

Figure 2 shows the analysis of the first 20 aminoterminal amino acids.

Example 6: Neutrophil-activating effect of NSA-1/NAP-2

Neutrophils were isolated from human blood, suspended in PBS/BSA and then used to assess the capacity of NSA-1/NAP-2 to induce elastase release using the microtiter plate assay method of Devaid and Baggiolini (1987). NSA-1/NAP-2 induced the selective release of elastase in a concentration-dependent manner. The concentration dependance was similar to that observed with NAF/NAP-1 and the potency was about half of that of NAF and about one third of that of fMLP.

Figure 3 shows the concentration-dependent activity of NSA-1/NAP-2.

PART II: CLONING OF cDNA CODING FOR CTAP-III FROM A HUMAN PLATELET-DERIVED λgt11 EXPRESSION LIBRARY

Example 7:

a) Isolation and washing of platelets.

Platelets were isolated from citrate-treated blood by centrifugation at 160 g for ten minutes to give platelet-rich plasma (PRP) and by a further centrifugation step at 1100 g for ten minutes to give a platelet pellet. The platelets were then washed twice with 30 mmol/l glucose, 120 mmol/l NaCl, 129 mmol/l sodium citrate, 10 mmol/l EDTA pH 6.5, and once with 10 mmol/l Tris/HCl, 154 mmol/l NaCl, 10 mmol/l EDTA pH 7.4.

b) Human megakaryocytes

These were isolated from the peripheral blood of a patient with megakaryoblastic leukemia by Ficoll-metrizoate gradient density centrifugation. The megakaryoblastic phenotype of these leukemic cells is based on their reactivity with monoclonal antibodies (MoAbs) to platelet GPIIb, the GPIIb/GPIIIa complex and (vWF) von Willebrand factor. A cDNA probe for platelet GPIb also gave a positive signal with a messenger RNA (mRNA) of 2.4 kb (same size as in platelets) in Northern blotting with mRNA from these cells.

c) Preparation of antibodies

Washed platelets were solubilized in 1 % Triton X-114 in the presence of N-ethyl maleimide (NEM) and phenylmethane sulfonyl fluoride (PMSF. dissolved in methanol) at a final concentration of 2 mmol/l each and phase partition was carried out as described in Biochim. Biophys. Acta 778 (1984) 463. The aqueous phase was used for AcA-34 Ultrogel (LKB) exclusion chromatography in 0.1 % sodium dodecyl sulfate (DDS), 0.1 mol/l NH_4HCO_3, pH 7.4. A fraction containing proteins in the 8- to 9- Kd range, as estimated by sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for immunization of rabbits.

d) Immunoblotting

Degranulation products were obtained from the supernatant of washed platelets stimulated with thrombin (2 U/4 x 10^9/ml, five minutes, 37°C) and treated with 2 mmol/l PMSF and 2 mmol/l NEM. They were solubilized in 1 % SDS, reduced with 0.1 % dithiothreitol (DTT), and separated by 20 % SDS-PAGE followed by electrophoretic transfer to nitrocellulose (BA 85, Schleicher & Schuell, Feldbach, Switzerland) with a semidry electroblotter at 150 mA for 90 minutes. After incubation with an anti-CTAP-III rabbit polyclonal antiserum, a goat antirabbit second antibody coupled to alkaline phosphatase (Bio-Rad Laboratories, Glattbrugg, Switzerland) was used for staining with the substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

e) Construction of the cDNA expression library

Total RNA of platelets from 180 l blood was prepared using the guanidine hydrochloride procedure. PolyA mRNA was isolated by oligo(dT) cellulose (Pharmacia, Uppsala, Sweden) affinity chromatography. First strand was synthesized using oligo(dT) primers (Pharmacia P-L Biochemicals) and reverse transcriptase (Bethesda Research Laboratories, Gibco, Basel, Switzerland). The second strand was prepared with RNase H (New England Biolabs, Schwalbach bei Frankfurt, FRG) and Escherichia coli DNA polymerase I (Boehringer Mannheim, Rotkreuz, Switzerland). After producing blunt ends with T4 DNA polymerase and Klenow enzyme, EcoRI methylation, and ligation to EcoRI linkers, the resulting cDNA was packaged in λgt11 packaging extract (Gigapack Gold, Stratagene, San Diego, CA) and amplified in E.coli y1088.

f) Library screening and characterization of positive clones

The platelet λgt11 cDNA expression library was screened by standard method with the polyclonal rabbit antiserum. E.coli-specific antibodies were removed by immunoadsorption on nitrocellulose-bound lysate of E.coli BNN97. Positive recombinant phages were detected by the same second antibody and chromogenic substrates as for immunoblotting. After purification, λ-DNA was digested with EcoRI (Boehringer Mannheim) and the inserts isolated by 0.8 % agarose gel electrophoresis and electroelution in a Bio-Trap (Schleicher & Schuell). The DNA was precipitated with ethanol and ligated to 5 ng EcoRI linearized M13 Bluescript (Stratagene) using T4 ligase (Bio-Lab) and transfected into E.coli JM 101. White colonies were tested for positive recombinant plasmids by the alkaline extraction procedure and single stranded cDNA templates were prepared by infection with M13KO7 helper phage. The DNA sequence of both strands was determined by the dideoxy-chain termination method using the Sequenase Kit (United States Biochemical Corporation and ^{35}S α-labeled dATP (New England Nuclear, Du Pont).

g) Northern blot analysis

Following electrophoresis in a 1 % agarose gel, mRNA was blotted onto Hybond N (TM) (Amersham) and was then hybridized with a sulfonylated cDNA probe at 68°C for 18 hours in 4 x SSPE, 0.1 % $Na_4P_2O_7$, 0.2 % SDS, 0.5 mg/ml heparin containing 100 µg/ml salmon sperm DNA. After washing twice for five minutes in 1 x SSPE, 1 % SDS, 0.1 % $Na_4P_2O_7$ at room temperature and twice for 30 minutes in 0.2 x SSPE, 0.1 % SDS, 0.1 % $Na_4P_2O_7$ at 68°C, the corresponding mRNA was detected by immunostaining with a murine monoclonal antibody (MoAB; Sigma, Deisenhofen, FRG) against sulfonylated DNA and a goat antimouse IgG second antibody conjugated to alkaline phosphatase. The substrates were the same as above.

h) Results

Polyclonal antibodies produced by immunization of rabbits with a gel filtration fraction containing platelet water-soluble proteins in the 8 to 9 kd range showed a high avidity and specificity on immunoblots and were used for screening a platelet-derived λgt11 cDNA expression library. Two positive clones, from 100 000 initially screened recombinant phages, were plaque purified, and the two internal EcoRI fragments were subcloned in M13 Bluescript^{RTM}. Nucleotide sequences of both strands were determined by the dideoxy-chain termination method. A full-length cDNA clone (λCI) of 690 base pairs was obtained (Figure 5) containing a 5′ non-coding region of 66 base pairs, an open reading frame coding for a protein of 128 amino acid residues (13 894 da) and a 3′ non-coding region containing the termination codon (TAA), the putative polyadenylation signal, and the polyA tail. A second clone (λC2), starting at the relative position -9, showed an identical nucleotide sequence.

The consensus sequence for initiation of translation in eukaryotic mRNA (5′ CCACCAUGA 3′) begins at nucleotide -5.

Northern blot hybridization of mRNA from a megakaryocytic leukemia cell line, megakaryocytes, and platelets but not from a hepatocyte control gave a positive signal at approximately 0.8 kb with the mRNA of the CTAP-III precursor, suggesting that the corresponding cDNA is full length. No signal was obtained with HEL mRNA, possibly due to lover sensitivity of the nonradioactive labeling method for the cDNA probe and/or the lov CTAP-III-specific mRNA content of the HEL cell line used.

The deduced amino acid sequence (Figure 5) is identical to the well-established sequence of human platelet CTAP-III. The amino terminus of CTAP-III is at amino acid position 44 of the translated sequence, and the beginning of its mitogenically inactive plasmin or trypsin degradation product β-thromboglobulin is four residues downstream at position 48. A precursor of these proteins, PBP, has

been shown to share the first ten residues of CTAP-III, but its amino terminus is nine residues upstream at position 35. All known structural information on these three proteins fits exactly the amino acid sequence derived from the coding cDNA clone, providing strong evidence for a single precursor for all three proteins.

Thus, the 34 amino acid leader sequence of CTAP-III shown in Figure 5 is exceptionally long, differs from classical signal peptides, and is probably responsible for targeting the protein to the α-granules of maturing megakaryocytes.

C L A I M S :

1. Substantially pure neutrophil-stimulating activity-1 (NSA-1) or, synonymously, neutrophil-activating peptide-2 (NAP-2) having the following 70 amino acid sequence:

1 5 10
Ala-Glu-Leu-Arg-Cys-Met-Cys-Ile-Lys-Thr-

11 15 20
Thr-Ser-Gly-Ile-His-Pro-Lys-Asn-Ile-Gln-

21 25 30
Ser-Leu-Glu-Val-Ile-Gly-Lys-Gly-Thr-His-

31 35 40
Cys-Asn-Gln-Val-Glu-Val-Ile-Ala-Thr-Leu-

41 45 50
Lys-Asp-Gly-Arg-Lys-Ile-Cys-Leu-Asp-Pro-

51 55 60
Asp-Ala-Pro-Arg-Ile-Lys-Lys-Ile-Val-Gln-

61 65 70
Lys-Lys-Leu-Ala-Gly-Asp-Glu-Ser-Ala-Asp

or a functional variant thereof having the above sequence preceded by, respectively,

Asp-Leu-Tyr-,
Ser-Asp-Leu-Tyr- or
Asp-Ser-Asp-Leu-Tyr-.

2. A factor according to claim 1 having an apparent molecular weight of approximately 6500 upon 20 % urea-SDS polyacrylamide gel electrophoresis and an apparent isoelectric point of about 8.3.

3. A process for the preparation of neutrophil-stimulating activity-1 (NSA-1) or, synonymously, neutrophil-activating peptide-2 (NAP-2) as defined in claim 1 which comprises purification from culture fluids of stimulated blood leukocytes and/or platelets by phosphocellulose chromatography and reversed-phase chromatography.

4. A process for the preparation of a factor as defined in claim 1 or of biologically active β-TG, CTAP-III or PBP which comprises cloning a corresponding gene including the natural leader sequence as defined in claim 7, expressing the gene in a suitable host and appropriately recovering the peptide product, if indicated, for preparing a factor as defined in claim 1, using an appropriate protease to cleave the resultant β-TG, CTAP-III or PBP.

5. A pharmaceutical composition comprising a factor according to claim 1 together with a pharmaceutically acceptable carrier or diluent.

6. A factor according to claim 1 for use as a pharmaceutical.

7. The leader peptide having the following amino acid sequence:

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1                   5                   10
MET-Ser-Leu-Arg-Leu-Asp-Thr-Thr-Pro-Ser-
11                  15                  20
Cys-Asn-Ser-Ala-Arg-Pro-Leu-His-Ala-Leu-
21                  25                  30
Gln-Val-Leu-Leu-Leu-Leu-Ser-Leu-Leu-Leu-
31
Thr-Ala-Leu-Ala-
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